

Cytologic Markers from Mammary Tissue of Women at Increased Risk of
Breast Cancer Development: Preliminary Analyses

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Introduction

Breast cancer originates in various components of the breast most commonly in the epithelial layer of the duct system or less frequently in the lobules. The tissue that surrounds and supports the lobules and duct system is comprised of adipose and stromal connective tissue. Lymphatic vessels are present throughout the breast with collections of lymph nodes in the axilla and substernal border

It is essentially unknown how a breast cancer begins or what triggers must be present to enable its growth. These gaps in understanding hinder the ability to identify and develop reliable and consistent biomarkers to help predict whether or not a woman will develop a cancer long before the actual tumor forms. Progress has been made towards the understanding of the pre-breast cancer microenvironment. Biomarkers that reflect the characteristics of the breast microenvironment potentially enable researchers to predict a woman's short-term risk for breast cancer and provide her with more information than what is obtained from a mammogram, Novel methods to obtain biomarkers include random periareolar fine needle aspiration (RPFNA).

This study was a review of RPFNA results with examination of each woman's RPFNA score, Masood scoring system descriptors, and indicators of inflammation. Preliminary findings support that the evidence of inflammation in RPFNA samples may be a strong indicator of breast cancer development.

Background/Significance

Random Periareolar Fine Needle Aspiration (RPFNA)

Random periareolar fine-needle aspiration (RPFNA) is a novel technique that combines fine needle aspiration with cytopathology in an effort to identify reliable and

accurate biomarkers to predict who may, and may not form a breast cancer. RPFNA obtains repeated samples of the breast tissue from women who have been identified as being at high-risk for the development of breast cancer. A cytopathologist is responsible for evaluating the tissue samples for cellular characteristics and presence of biomarkers. One biomarker of interest is inflammation, which has already demonstrated to be a significant indicator of breast cancer development. Macrophages are also of interest and are considered the modulators of breast cancer progression.

Breast Composition

The breast is composed of milk glands (lobules) that produce milk, ducts that transport milk to the nipple, the nipple, the areola (the pigmented area surrounding the nipple), connective tissue surrounding the lobules and ducts, and finally, fat. The lobules and ducts are located in the glandular tissues of the breast. The human breast begins to develop about six weeks gestation with breast tissue initially extending along the armpits all the way down to the groin (Imaginis, 2011). By nine weeks gestation, that tissue has regressed and formed two breast buds in the chest area of both males and females (Imaginis, 2011). In females, columns of cells grow inward from the buds and eventually become sweat glands with ducts leading to the nipple (Imaginis, 2011). Female breasts continue to grow when puberty is reached. The production of estrogen and progesterone are what stimulate this process, and glandular breast tissue begins to develop and the fat and fibrous tissue becomes more elastic (Imaginis, 2011).

Breast Cancer Screening

Mammography has been the gold standard for breast cancer screening and early detection, both currently and historically. It is a quick and fairly painless procedure that

allows radiologists to detect tumors before they are large enough to be palpated during a self-breast or clinical breast exam. Despite its wide acceptance, mammography does have limitations. Mammography fails to detect all breast cancers, specifically invasive lobular carcinomas and non-calcified ductal carcinoma in situ. Mammography has a false-negative rate in the range of twenty to thirty percent (Bartella, Smith, Dershaw, & Liberman, 2007). Other types of imaging include ultrasonography and are used to further differentiate lesions and masses detected by mammography. Biopsies are also a common adjunct to mammography to diagnose benign lesions and masses. In recent years, breast magnetic resonance imaging (MRI), originally used as a method of detecting ruptured breast implants, has become more accepted as a diagnostic tool due to its value in detecting breast abnormalities (Johnson, 2012). The potential benefits of MRI are attractive to clinicians: fewer re-excisions after breast conserving surgery, decreased local recurrence rates after excision, and earlier detection and treatment of contralateral breast cancer (Tejada-Berges, 2011).

Mammography alone, or in conjunction with ultrasound, was found to be insufficient for early diagnosis in women who are considered high risk, compared to breast MRI that had significantly higher sensitivity and was able to identify cancers at earlier stages (Kuhl, et al., 2005). Recommendations from the American Cancer Society state that if a woman's lifetime risk of developing breast cancer is estimated to be greater than 20% - 25%, MRI-based screening should be utilized (Saslow, et al., 2007). Currently, mammography is still the most commonly used diagnostic and screening tool, with ultrasound and MRI being used if deemed necessary by a physician.

Breast Cancer Risk

Most breast cancers are sporadic, or not inherited, but some are the result of inherited predisposition to the cancer, most commonly due to mutation of the tumor suppressor genes, BRCA1 and BRCA2 (King, et al., 2003). A woman is at highest risk of development of a cancer when she is found to be gene positive for BRCA genes,, the other risk factors include a positive family or personal history. In families with multiple cancer cases and the presence of BRCA1 and BRCA2 mutations, the estimated lifetime risk of breast cancer is greater than eighty percent (King, Marks, & Mandell, 2003).

A positive family history is more specifically defined as having multiple first- and second-degree relatives who have had breast or ovarian cancer. Of highest risk is a first degree relative that was diagnosed with breast cancer before age fifty, or a male relative with breast cancer. It has been shown that in some families, gene-positive women have up to an eighty-five percent lifetime risk of developing breast cancer (Bartella, et al., 2007). A positive family history of breast cancer has also been shown to almost double the risk of developing invasive breast cancer in a patient who is already diagnosed with atypical ductal hyperplasia (ADH) (Tejada-Berges, 2011).

Risk Assessment Tools

The Gail risk assessment tool was developed to project the probability of a woman developing breast cancer based on personal and family history and is referred to as the patient's "lifetime risk" of developing breast cancer. The items that Gail takes into account are age at first menarche, age at first live birth, current age, number of first-degree relatives with breast cancer, number of personal breast biopsies, and presence/absence of atypical hyperplasia.

The Breast Cancer Risk Assessment Tool (BCRAT) is a more frequently used and modified version of the Gail model (Cyr, et al., 2011). This incarnation of this revised Gail model takes into account the same factors as the original Gail, but it is modified to have improved validity in African-American women (Cyr, 2011).

Random Periareolar Fine Needle Aspiration

Random periareolar fine-needle aspiration (RPFNA) is a novel method of sampling breast tissue that is gaining acceptance in the clinical world. RPFNA is a highly reproducible measure of breast cytology (Ibarra-Drendall, et al., 2009). Studies have shown that when four to five passes were taken per site on the breast, adequate cytology for morphologic assessment was achieved in 94% of the women (Fabian, et al., 2000). Reproducibility of RPFNA was based on one clinician being able to produce consistent samples, but something that has not been looked at is how reproducible RPFNA is between institutions and clinicians; if a patient had RPFNA performed at two different institutions by two different clinicians, would the findings be the same? This unknown factor may be a large factor in clinicians not wanting to use it in practice; more studies need to be performed.

Though research has allowed us to be able to identify and treat premalignant lesions, there has not yet been a decrease in the incidence of invasive breast cancers (Tejada-Berges, 2011). This could be due to the fact that as of yet, no cytologic or clinical parameter allows for clear identification of women at greatest risk of developing a cancer. Biomarkers become of interest because of this reason. Biomarkers are the characteristics of the breast microenvironment that will potentially allow us to predict short-term risk for breast cancer and identify the women who would be most likely to benefit from prevention.

We hope to pinpoint a characteristic of the microenvironment of the breast that would allow us to identify the highest-risk women.

Inflammation

Inflammation is a biomarker of interest as it has already been found to have a molecular link with cancer. At this time, epidemiological data indicate that over a quarter of all cancers are related to chronic infections or other types of unresolved inflammation (Vendramini-Costa, & Carvalho, 2012). It has been hypothesized that cellular environments that drive the initiation and development of carcinogenesis are promoted by an inflammatory milieu (Mantovani, 2009). Chronic inflammation acts as a regulator of tumor progression and promotion through mechanisms that include accelerated cell proliferation, evasion from apoptosis, and enhanced angiogenesis and metastasis (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006). There is an inflammatory component in the microenvironment of most neoplastic tissues, even ones that are not caused by an inflammatory process (Mantovani, Schioppa, Porta, Allavena, & Sica, 2006). Inflammatory diseases, like inflammatory bowel disease, increase the risk of cancer. In tumors and cancers that are epidemiologically unrelated to inflammatory conditions, the oncogenes are activated, and initiate the production and recruitments of inflammatory molecules and cells (Mantovani, 2009).

Macrophages

Macrophages are key players in the inflammatory process, which labels them as another potential biomarker of interest for breast cancer. The functions of macrophages in tumors are the same functions that are seen in everyday wound healing and chronic inflammation. This fact supports a notion that tumors are wounds that never heal and

allows us to infer that inflammation may be important in the development and promotion of tumors.

The presence of stromal, or connective tissue, fragments indicates that a cancer is more apt to be invasive. The abundance of macrophages in the tumor microenvironment is correlated with poor prognosis (Pollard, 2008). Using a meta-analysis, one study showed that an increased macrophage density was associated with poor prognosis in greater than 80% of the cases (Bingle, Brown, & Lewis, 2002). Macrophages promote tumor cell invasion in the following ways: 1) by producing EGF ligands that stimulate tumor cell motility, 2) by inducing the formation of new vessels that are sheathed in collagen and therefore, focuses the migration of tumor cells to the area (Pollard, 2008). These macrophage-promoted activities of increased angiogenesis will result in increased metastatic capacity of the tumor (Pollard, 2008).

More specifically, tumor-assisted macrophages (TAM) are able to affect different parts of neoplastic tissues because they are the major inflammatory component of the stroma, or connective tissue, of many tumors (Mantovani, et al., 2006). TAMs have been shown to produce angiogenic factors, stromal breakdown factors, and to suppress adaptive immunity, all three of which promote the progression of tumors (Mukhtar, et al., 2012). In both mouse and human studies, the majority of macrophages are found in the stroma that surrounds the tumor (Pollard, 2008). One study postulated that the therapeutic targeting of macrophage-derived mediators may lead to new therapeutic strategies against the invasion and metastasis of cancer (Mantovani, 2006). It is important to identify what TAMs are specifically contributing to the growth of tumors so that they can potentially be used as prognostic markers and targets of treatment (Mukhtar, et al., 2012).

Columnar Cell Lesions

More recently, columnar cell lesions have gained increased attention for their potential link to the development of breast cancer. These lesions, which tend to calcify, are getting recognized more often as a result of mammography screening and core needle biopsies (Verschuur-Maes, 2012). Core needle biopsies tend to diagnose the non-calcified columnar cell lesions that mammography miss due to their not being calcified. Columnar cell lesions have been seen to have associations with atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), lobular carcinoma in situ, and low-grade invasive carcinomas when looked at in surgical excision specimens (Simpson, et al., 2005). Columnar cell lesions, especially those associated with atypia, have been increasingly regarded as putative precursors to low-grade carcinomas (Sinn, 2009). More research is needed on these lesions to be able to determine their usefulness as a biomarker for breast cancer treatment.

Family History

There are many limitations in the research for biological markers of high-risk status. A large area of uncertainty is in identifying patients considered to be high-risk for breast cancer development. While we do have BRCA1 and BRCA2 mutations and family and personal history as means of identifying someone as being high risk, we have to consider the possibility of certain people not knowing their family history or misreporting it to their healthcare provider. Even if a patient is determined to be high-risk, we still don't know if they will develop a cancer or not. We need more research, like this study, to determine if there are ways to determine if a person will develop a cancer or not.

Methods

This institutional review board-approved study was a retrospective review of 104 subjects with 499 RPFNA samples. Subjects were selected for the study from the high-risk patient population at a Midwestern Comprehensive Breast Center. The initial evaluation of the potential subjects consisted of three criteria: 1) breast patient information form, 2) clinical breast exam, and 3) mammogram. The patient's chart was reviewed first in order to determine if the patient qualified for inclusion and exclusion criteria set forth by the study. Eligibility was based on the lack of suspicious findings on the potential subjects' mammograms and clinical breast exams. Evaluation of the mammograms also determined if the patient has enough breast density for collection of a sample with an adequate amount of epithelial cells for evaluation.

The procedure for collecting the breast tissue samples was random periareolar fine-needle aspiration (RPFNA). This procedure involved taking samples from two areas on each of the breasts: 10:00 and 2:00 positions. The areas were anesthetized with lidocaine prior to the introduction of the needle. The goal of the aspiration was to withdraw approximately 10^5 cells and 0.5 cc of breast fluid from the areas. For each area, four separate needles were used and two of the four samples from each area were placed in one of two collection tubes containing a mixture of modified Cytolyt and 1% formalin. There were separate collection tubes for each breast.

Cytopathologic Evaluation

Tissue samples were then sent out for evaluation by a single cytopathologist for the study. Every sample from the study was evaluated by the same cytopathologist so that there was consistency in the scoring of the samples. The cytopathologist used Masood's Scoring Index, which was developed specifically for the evaluation of breast tissue, to

assign each sample a score. This score was based on six individual descriptors: cellular arrangement, cellular pleomorphism, myoepithelial cells, anisonucleosis, nucleoli, and chromatin clumping. A sample receives a score of one to four in each of these categories to receive a total score that is anywhere between six and twenty-four. Scores from six to eight indicate nonproliferative breast disease, nine to fourteen indicates proliferative breast disease without atypia, fifteen to seventeen indicates proliferative breast disease with atypia, and scores of eighteen to twenty-four indicate carcinoma in situ and invasive cancer.

The aforementioned importance of the patient's breast being dense enough to aspirate a sample with an adequate number of epithelial cells becomes important in the scoring of the samples. When looking at the sample on a wet mount slide, the cytopathologist looks at the sample as a whole and estimates the total number of epithelial cells present and if there are stromal fragments or macrophages present. If there are epithelial cells present in the sample, the cytopathologist selects the largest clump of cells for evaluation and scoring of the entire sample. Therefore, the more epithelial cells present, the more accurate the score will be of the actual breast environment.

Based on the Masood scores of the tissue samples, patients are eligible to come back for either a six-month or one-year follow-up RPFNA visit. If a patient receives a score of fourteen or below, they can come back in one year, if the score is fifteen or above, we request six months. Return visits are done on a voluntary basis for the patients; some may only have the first visit, some return upwards of eight times.

Results

This study is still ongoing, so only preliminary analyses of the data have been performed. A one-way ANOVA test was used for the preliminary analyses. This type of statistical testing was used because it looks more at causation between data points, such as breast cancer and presence of macrophages in the breast microenvironment, rather than just a basic correlation.

The presence or absence of inflammation between women with and without breast cancer was tested and a statistically significant difference $F(2,466) = 3.55, p = 0.029$ indicating that “inflammation” accurately designated a patient with breast cancer. A one-way ANOVA was also used to test the presence or absence of inflammation between breasts with and without cancer, but it was not statistically significant $F(1,467) = 1.03, p = 0.311$. The finding in this test suggests that the “field effect” is evident in pooled samples versus individual breasts per laterality. One last ANOVA was fitted to the data to determine if there was a statistically significant difference with other descriptors related to breast cancer. No statistically significant differences were found between or within groups for: macrophages $F(2,486) = 0.171, p = 0.843$; stromal fragments $F(2,486) = 0.837, p = 0.434$; or both $F(2,486) = 0.084, p = 0.919$.

Discussion

Preliminary analyses of the data collected thus far for the study shows that the presence or absence of inflammation is a strong indicator of the presence or absence of breast cancer in the patient. RPFNA currently remains under investigation, though it does hold promise of being the “pap smear” of the breast for women at high risk of developing breast cancer.

Implications

The identification of biomarkers could potentially allow us to treat women at high risk for breast cancer earlier, thus decreasing the incidence of invasive breast cancer. There are many potential biomarkers to be considered, and most are already the focus of studies aimed establishing earlier treatment strategies.

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